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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 9/127

A1

(11) International Publication Number: WO 92/14448

(43) International Publication Date: 3 September 1992 (03.09.92)

(21) International Application Number: PCT/US91/08114

(22) International Filing Date: 30 October 1991 (30.10.91)

(30) Priority data:
655,576 14 February 1991 (14.02.91) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BINDING OF GROWTH HORMONE TO LIPOSOMES

(57) Abstract

Crosslinking reagents glutaraldehyde and ethylene glycol diglycidyl ether have been used to bind epidermal growth factor ("EGF") to liposomal surfaces without utilizing the disulfide bridge linkage of EGF and, therefore, not adversely affecting the functionality of EGF. The crosslinking occurs through the amine residues offered by EGF and liposomes having phosphatidylethanolamine. The resulting EGF-modified liposomes offer potential as a microscopic drug delivery system.

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BINDING OF GROWTH HORMONE TO LIPOSOMES

BACKGROUND OF THE INVENTION

The present invention relates to the preparation of microscopic drug delivery systems (MDDS) utilizing drug-encapsulating bioadhesive liposomes.

Microscopic drug delivery systems (MDDS) have been developed for improved drug administration relative to administration of drugs in their free form. Drug-loaded MDDS can perform as sustained or controlled release drug depots. By providing a mutual protection of the drug and the biological environment, MDDS reduces drug degradation or inactivation. As a system for controlled release of a drug, MDDS improves drug efficacy and allows reduction in the frequency of dosing. Since the pharmacokinetics of free drug release from depots of MDDS are different than from directly-administered drug, MDDS provides an additional measure to reduce toxicity and undesirable side effects.

MDDS is divided into two basic classes: particulate systems, such as cells, microspheres, viral envelopes and liposomes; or nonparticulate systems which are macromolecules such as proteins or synthetic polymers. Liposomes have been studied as drug carriers and offer a range of advantages relative to other MDDS systems. Composed of naturally-occurring materials which are biocompatible and biodegradable, liposomes are used to encapsulate biologically active materials for a variety of purposes. Having a variety of layers, sizes, surface charges and compositions, numerous procedures for liposomal preparation and for drug encapsulation within them have been developed, some of which have been scaled up to industrial levels.

Liposomes can be designed to act as sustained release drug depots and, in certain applications, aid drug access across cell membranes. Their ability to protect encapsulated drugs and various other characteristics make liposomes a popular choice in developing MDDS, with respect to the previous practices of free drug administration.

Despite the advantages offered, utilization of drug-encapsulating liposomes does pose some difficulties. For example, liposomes as MDDS have limited targeting abilities, limited retention and stability in circulation, potential toxicity upon chronic administration and inability to extravasate. In recent years, successful attempts have been made to bind different substances to liposomes. For example, binding of chymotrypsin to liposomes has been studied as a model for binding substances to liposomal surfaces. Recognizing substances, including antibodies, glycoproteins and lectins have been bound to liposomal surfaces in an attempt to confer target specificity to the liposomes. Concentrating on systemic applications and in vivo studies, these previous efforts discuss methods of binding recognizing substances with liposomes and the effectiveness of such modified liposomes. Although the bonding of these recognizing substances to liposomes occurred, the resulting modified liposomes did not perform as hoped, particularly during in vivo studies. Other difficulties are presented when utilizing these recognizing substances. For example, antibodies can be patient specific and, therefore, add cost to the drug therapy.

It has also been previously learned that liposomes can be modified by binding epidermal growth factor (EGF) to the liposomal surface as a recognizing substance. EGF stimulates

cell growth and proliferation through the interaction with the EGF receptor. The EGF receptors are distributed on the cell surface of various organs and are present in burns, wounds, and other designated targets of MDDS such as ocular, dermal and tumors. Accordingly, EGF-modified liposomes potentially offer efficiency as drug carriers to target sites expressing the EGF receptors.

EGF is a polypeptide. It has been reported that the biological activity of EGF is dependent upon the conservation of the native conformation of EGF, to which the disulfide bonds are critical. Previous work in binding EGF to liposomes has capitalized on the existence of the disulfide bonds. Specifically, EGF has been bound to the liposomal surface by the disulfide bridge linkage using a heterobifunctional crosslinking reagent, N-hydroxysuccinimidyl-3-(2-pyridyldithio) propionate. Although the resulting EGF-modified liposomes have been suggested as potential drug carriers for systemic applications, the complex chemistry of this process results in byproducts whose effect on drug delivery and toxicity are unknown.

EGF is bound covalently to discrete sites on the liposome surfaces. The number and surface density of these sites will be dictated by the liposome formulation and the liposome type. The liposomal surfaces may also have sites for noncovalent association. Covalent binding is essential as noncovalent binding might result in dissociation of EGF from the liposomes at the site of administration since the liposomes and the bioadhesive counterparts of the target site (that is, the bioadhesive matter) compete for EGF. Such dissociation would

reverse the administered modified liposomes into regular, non-modified liposomes, thereby defeating the purpose of administration of the modified liposomes.

To form covalent conjugates of recognizing substances and liposomes, crosslinking reagents have been studied for effectiveness and biocompatibility. One such reagent is glutaraldehyde (GAD). Through the complex chemistry of crosslinking by GAD, linkage of the amine residues of the recognizing substance and liposomes is established. Previous efforts have studied the binding of chymotrypsin and liposomes with GAD as the crosslinking reagent.

SUMMARY OF INVENTION

According to the present invention, methodologies have been developed and crosslinking reagents have been identified to bind growth hormones, preferably epidermal growth factor (EGF), to liposomal surfaces. More specifically, crosslinking reagents have been identified which crosslink amine residues on the liposomal surfaces to the residues offered by EGF. The crosslinking reagents include glutaraldehyde (GAD) and bifunctional oxirane (OXR), preferably ethylene glycol diglycidyl ether (EGDE). By modifying regular liposomes through covalent bonding certain growth hormones to the liposomal surface, the growth hormones are recognizing substances which can be utilized as an adhesive or glue to attach the modified liposome onto a target area. These "bioadhesive" liposomes offer potential advantages as a MDDS for the administration of drugs which is further disclosed in my concurrently filed applications.

DETAILED DESCRIPTION

According to the present invention, growth hormones, preferably EGF, have been covalently bound to liposomal surfaces as a recognizing substance through the crosslinking of amine residues. Liposomes, in particular, multilamellar vesicles (MLV), microemulsified liposomes (MEL) or large unilamellar vesicles (LUVET), each containing phosphatidylethanolamine (PE), have been prepared by established procedures. Although urogasterone and EGF are recognized as biological equivalents, both purified urogasterone or EGF mouse were used as recognizing substances, with the results reported in Examples 1 and 2. In the specification and claims the term "EGF" means either urogasterone or epidermal growth factor regardless of the source. Growth hormone was linked to the PE-liposomes.

In the Examples, the EGF and lipids are assayed by traces of radioactive or fluorescent labels included in each formulation. Alternatively, the lipids are assayed by colorimetric methods. The determination of the non-labeled and the fluorescent-labeled EGF can also be performed by the Lowry procedure previously reported.

The "level of covalent binding" as reported in the Examples and Tables 1 and 2, is defined as the quantity of ligand or EGF bound to a given quantity of lipid in the final product since the most accurate quantitative measure of liposomes is in terms of lipid quantities. For a given lipid quantity, different liposome types will yield different quantities of liposome. Therefore, similar initial ratios of EGF to lipid for different liposome types should not be expected to yield the same level of binding. Another factor

which would yield different results for different liposomes even under the same initial EGF to lipid ratios, is the differences in particle size, therefore in curvature, number and accessibility of PE sites on the surface of the liposome. Therefore, comparisons among liposome types should be avoided.

The effects of the increase in the EGF/lipid ratios in the presence of a crosslinking reagent are shown in Tables 1 and 2. Generally, an increase in the level of binding occurs with the increase in initial EGF/lipid ratios regardless of which crosslinking reagent is used. At the lower end of the EGF/lipid ratios, the level of covalent binding increases significantly. Beyond initial concentration ratios of 25 ng EGF/uMoles lipid, the increase of binding is less significant. Noncovalently bound product is removed as excess unreacted material and does not appear in the reported results.

Example One

EGF is added to a PE-liposome sample and the mixture is buffered by a phosphate buffer saline solution (PBS) to pH of 7.2. Concentration ratios of EGF to lipid are shown in Table 1. Aliquots from a 25% solution of the crosslinking reagent GAD are added at a ratio of 10ul per 1 ml EGF/PE-liposome mixture. Incubation for a desired period is completed at either room temperature without stirring or at 37°C with stirring. Depending upon the liposome used, excess unreacted material was removed through centrifugation and washings, column chromatography or dialysis against PBS.

TABLE 1

EGF-LIPOSOME CROSSLINKING BY GAD

	ng EGF/uMOLES LIPID (a)	
LIPOSOME TYPE	INITIAL	FINAL
MLV	0.080	0.009
MLV	0.309	0.006
MLV	0.347	0.016
MEL	0.071	0.004
MEL	0.106	0.009
MEL	0.141	0.025
LUVET	0.016	0.003

(a) EGF assayed by a radioactive tracer.

Example Two

EGF is crosslinked with PE-liposome samples following the same procedure as in Example 1. Concentration ratios of labeled EGF to lipid are shown in Table 2.

TABLE 2

EGF-LIPOSOME CROSSLINKING BY GAD

	ng EGF/uMOLES LIPID (a)	
LIPOSOME TYPE	INITIAL	FINAL
MLV	0.26	0.07
MLV	0.78	0.16
MLV	1.60	0.21
MLV	6.00	0.31
MLV	24.70	0.35

(a) EGF assayed by a fluorescent tracer.

Example Three

Reaction mixtures of EGF and PE-liposomes were prepared as above and buffered by PBS to pH 7 or by 0.5N carbonate buffer to pH 9. Concentrations ratios of EGF to lipid are shown in Table 3. The crosslinking reagent EGDE was added in 0.2 - 1.0 ml volumes to buffered reaction mixtures of 2.5 - 3.0 ml volumes. Incubation periods were completed for 10-24 hours at 37°C with stirring. Depending upon the liposome used, excess unreacted material was removed through ultracentrifugations and washings or dialysis against PBS.

TABLE 3

EGF-LIPOSOME CROSSLINKING BY EGDE

	ng/EGF/uMOLE LIPID (a)			
LIPOSOME TYPE	INITIAL	FINAL	pH	mg EGDE
MLV (b)	0.45	0.0078	9	500
MLV	3.72	0.90	9	500
MEL	0.10	0.012	9	500
MEL	0.10	0.0098	9	1000
MEL (a)	0.12	0.0022	7	200
MEL	1.78	0.47	9	500

(a) EGF assayed by a radioactive tracer.

(b) Initial ratios were increased by decreasing lipid concentration.

From these results, the preferred pH of 9 and quantity of crosslinking reagent of 500 mg has been determined.

While the preferred embodiments have been described, various modifications and substitutions may be made without departing from the scope of the invention. For example, the mouse EGF and human urogasterone used in the disclosed examples could be substituted with growth factors from other natural or synthetic sources. Accordingly, it is to be understood that the invention has been described by way of illustration and not limitation.

CLAIMS

What I claim is:

1. A process for producing modified liposomes comprising the steps of:

(a) providing a reaction vessel containing a liposome component having liposome and phosphatidylethanolamine;

(b) adding a recognizing substance component of a growth factor to the reaction vessel;

(c) buffering the reaction mixture of the liposome and recognizing substance components;

(d) adding a crosslinking reagent to the buffered reaction mixture; and

(e) allowing the buffered reaction mixture with reagent to incubate for a period of time sufficient for the modified liposome to form.

2. The process of claim 1 wherein the liposome is selected from the group consisting of multilamellar vesicles, microemulsified liposomes and large unilamellar vesicles.

3. The process of claim 1 wherein the growth factor is epidermal growth factor.

4. The process of claim 1 wherein the growth factor is urogasterone.

5. The process of claim 1 wherein the crosslinking reagent is glutaraldehyde.

6. The process of claim 1 wherein the crosslinking reagent is a bifunctional oxirane.

7. The process of claim 1 where in the crosslinking reagent is ethylene glycol diglycidyl ether.

8. The process of claim 1 wherein the recognizing substance is labeled with a fluorescent marker.

9. The process of claim 1 wherein the recognizing substance is labeled witn a iodinated marker.

10. A modified liposome produced according to the method of claim 1.

11. A process for covalently bonding a recognizing substance to a liposome comprising the steps of:

(a) providing a reaction vessel containing a liposome component having liposome and phosphatidylethanolamine;

(b) adding a recognizing substance component of growth factor to the reaction vessel;

(c) buffering the reaction mixture of the liposome and recognizing substance components;

(d) adding a crosslinking reagent to the buffered reaction mixture;

(e) allowing the buffered reaction mixture with reagent to incubate for a period of time sufficient for the covalent bonding of amine residues of the recognizing substance and liposome components; and

(f) separation and removal of covalently bound recognizing substance liposome components from excess unreacted and noncovalently bound materials.

12. The process of claim 11 wherein the liposome is selected from the group consisting of multilamellar vesicles, microemulsified liposomes and large unilamellar vesicles.

13. The process of claim 11 wherein the growth factor is epidermal growth factor.

14. The process of claim 11 wherein the growth factor is urogasterone.

15. The process of claim 11 wherein the crosslinking reagent is glutaraldehyde.

16. The process of claim 11 wherein the crosslinking reagent is a bifunctional oxirane.

17. The process of claim 11 where in the crosslinking reagent is ethylene glycol diglycidyl ether.

18. The process of claim 11 wherein the recognizing substance component is labeled with a fluorescent marker.

19. The process of claim 11 wherein the recognizing substance component is labeled with a iodinated marker.

20. A modified liposome produced according to the method of claim 11.

21. A modified liposome comprising a liposome component covalently linked to a recognizing substance component by a crosslinking reagent.

22. The modified liposome of claim 21 wherein the liposome component includes phosphatidylethanolamine.

23. The modified liposome of claim 21 wherein the liposome component is selected from the group consisting of multilamellar vesicles, microemulsified liposomes and large unilamellar vesicles.

24. The modified liposome of claim 21 wherein the recognizing substance component is a growth factor.

25. The modified liposome of claim 21 wherein the recognizing substance component is an epidermal growth factor.

26. The modified liposome of claim 21 wherein the recognizing substance component is urogasterone.

27. The modified liposome of claim 21 wherein the crosslinking reagent is glutaraldehyde.

28. The modified liposome of claim 21 wherein the crosslinking reagent is a bifunctional oxirane.

29. The modified liposome of claim 21 wherein the crosslinking reagent is ethylene glycol diglycidyl ether.

30. The modified liposome of claim 21 wherein the recognizing substance component is labeled with a fluorescent marker.

31. The modified liposome of claim 21 wherein the recognizing substance component is labeled with a iodinated marker.

32. The modified liposome of claim 21 wherein the crosslinking reagent is selected from a group of reagents linking amine residues of the liposome component and the recognizing substance component.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/08114

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K9/127

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 160, no. 2, 28 April 1989, DULUTH ,MINN. (US) pages 732 - 736; Y. ISHII ET AL.: 'preparation of egf labeled liposomes and their uptake by hepatocytes'	21-26, 30,31
Y	see the whole article	1-5, 8-15, 18-20, 26,27,32
Y	G. GREGORIADIS 'liposome technologie' 1984 , CRC PRESS, INC. , BOCA RATON, FLORIDA (US) see volume iii, pages 75-94; in particular pages 79-81, chapter a and pages 88-89	1-5, 8-15, 18-20, 26,27,32

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 JUNE 1992	23.06.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BENZ K.F. Benz

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WORLD PATENTS INDEX LATEST Week 8509, Derwent Publications Ltd., London, GB; AN 85-052381(09) & JP,A,60 008 326 (HITACHI CHEMICAL KK) 17 January 1985 see abstract	6,7,16, 17,28,29
A	WO,A,9 009 782 (LIPOSOME TECHNOLOGY, INC.) 7 September 1990 see the whole document; in particular page 4, line 14- page 5, line 27	1-32

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9108114 SA 57254

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 05/06/92
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9009782	07-09-90	US-A-	4944948	31-07-90
		US-A-	5064655	12-11-91
		AU-A-	5182590	26-09-90
		EP-A-	0460100	11-12-91

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